DEUTERIUM AND CARBON-13 TRACER STUDIES OF ETHANOL METABOLISM IN THE RAT BY $^{2}H,^{1}H$-DECOUPLED ^{13}C NUCLEAR MAGNETIC RESONANCE

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Received December 17,1973

SUMMARY: [1-^{13}C, 1,1-$^{2}H_2$] ethanol and [2,2,2-$^{2}H_3$] ethanol were administered to bile fistula rats. A new technique, $^{2}H,^{1}H$-decoupled ^{13}C nuclear magnetic resonance, was used in attempting to account for the distribution of the isotopic species along the steroid skeleton of 3-45 mg of isolated bile acids. The technique revealed ^{2}H incorporation at many carbon sites unambiguously, but has limitations as a quantitative ^{2}H assay at these levels of sample availability.

Interest in charting metabolic pathways by non-radioactive isotope incorporation in vivo has resulted in the exploration of new instrumental techniques to overcome the many orders of magnitude difference in detectability of ^{2}H, ^{13}C, ^{17}O, in comparison to their radioactive counterparts. ^{2}H-incorporation into metabolic products on the sub-nanogram level can be detected by the molecular ion m/e ratios in mass spectra (1), but the site of incorporation in the molecule is often unattainable by this technique. At larger sample levels, ^{1}H or ^{2}H nuclear magnetic resonance (nmr) may be expected to reveal incorporation sites, but only in relatively simple cases where attribution of individual resonances to specific nuclei is possible. The greatly increased resolution of ^{1}H-decoupled ^{13}C nmr has recently been shown to allow ^{2}H-site detection through ^{2}H-^{13}C isotopic shifts and coupling effects (2). We wish to report here a further improvement in this technique, that of simultaneous ^{2}H- and ^{1}H-decoupled ^{13}C nmr, to detect in vivo incorporation of ^{2}H from [1-^{13}C, 1,1-$^{2}H_2$] ethanol and [2,2,2-$^{2}H_3$] ethanol in bile acids of the rat. In a study of ethanol metabolism, this biosynthetic system has the advantage that labeled atoms are introduced in two separate pathways, i.e., via acetate and via reduced pyridine nucleotides. Furthermore, a relatively large quantity of product for analysis is obtained from a single animal. Parallel studies by gas chromatography-mass spectrometry are reported elsewhere (3).

MATERIALS AND METHODS

[1-^{13}C, 1,1-$^{2}H_2$] ethanol and [2,2,2-$^{2}H_3$] ethanol were synthesized from

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nominally 60% ^{13}C-enriched acetic acid, and 95% ^{2}H-enriched [2,2,2-$^{2}H_3$] acetic acid, respectively, and analyzed by gas chromatography-mass spectrometry of the benzoate. Male Sprague-Dawley rats weighing about 200 g were provided with bile fistulae and thin catheters for intraperitoneal injections. The labeled ethanol was administered in small aliquots (in saline) starting 48 hr after the operation, and concentration of ethanol in bile was monitored by gas chromatography. The volumes injected were adjusted to maintain a concentration of ethanol above 10 mM. The [1-^{13}C, 1,1-$^{2}H_2$] ethanol was administered to one rat and bile collected for 23 hr. The sample taken between 13 and 18 hr was used for analysis. [2,2,2-$^{2}H_3$] ethanol was administered to two rats and bile collected for 48 hr. The rats were kept in restraining cages and had free access to food pellets and saline.

Bile acids were extracted, saponified, methylated, and separated on hydroxyalkoxypropyl Sephadex or silicic acid (3,4). Aliquots were converted into trimethylsilyl ethers and heavy isotope content was determined using a 1% SE-30 column at 240°C in an LKB 9000 gas chromatography-mass spectrometry instrument, in conjunction with an incremental magnetic tape spectral recording system (5). Background subtraction, normalization, plotting and calculation of heavy isotope abundance was performed on an IBM 1800 computer (6). Methyl [3β-^{2}H] cholate, [3β,7β-$^{2}H_2$] cholate, [3β-^{2}H] chenodeoxycholate and [7β-^{2}H] chenodeoxycholate were synthesized by $NaB^{2}H_4$ reduction of the corresponding oxo-esters, for use as nmr standards. Other bile acid methyl esters were obtained commercially to assist in assigning ^{13}C nmr resonances.

A Varian XL-100-15 ^{13}C Fourier transform nmr spectrometer in conjunction with an XDS Sigma 7 computer system (7) was used to record the ^{13}C spectra. The nmr probe was fitted with an "external" pulse-modulated lock, which included a Cu^{++}-doped LiCl capillary, transmitter, preamplifier and 38.929 MHz phase locked crystal oscillator. A 15.361 MHz frequency modulated crystal oscillator and 10 w amplifier, coupled to the ^{13}C transmitter coils through a matching network, comprised the ^{2}H-decoupling system. Bile acid methyl ester samples (3-45 mg) were dissolved in 5 mm nmr tubes, and spectra recorded at 50°C using radio-frequency pulses placed in the center of the ^{13}C spectrum at 5 sec intervals. A 200 μsec A/D sampling rate and dual-phase detection (8) in conjunction with a 32-bit 16,384-word computer memory block afforded ^{13}C shift resolution of ±0.02 ppm, after 2000-15000 accumulated free-induction decays per channel and subsequent Fourier transform. A trace of tetramethylsilane was added to all samples to serve as an internal shift reference. The shift reagent Eu(FOD)$_3$ (Bio-Rad Laboratories) was used in chloroform solvent in some cases to assist in confirming ^{13}C spectral assignments.

RESULTS

^{13}C chemical shift assignments of unenriched methyl cholate and chenodeoxycholate in pyridine were made based upon comparisons of their spectra with those of deuterated or acetylated analogs, spectra of lithocholate and deoxycholate, known biosynthetic routes for carbon incorporation, LIS-reagent studies, published assignments (9) of other steroids, and off-resonance ^{1}H-decoupled spectra. Similar spectra in chloroform and 50% (v/v) pyridine-chloroform demonstrated a "scrambling" effect on the order of these assignments in their appearance from low to high fields, as has been reported elsewhere (10) for cholesterol, pyridine, and uridine. For example, in chloroform the C_{23} of methyl cholate shifts downfield by 0.8 ppm (when corrected for susceptibility differences and internal reference solvent dependencies) from its position in pyridine, while C_2 crosses it and shifts upfield by 0.4 ppm. The expectation that these spectral shifts would be helpful in uncovering ^{13}C-^{2}H isotope peaks hidden by nearby resonances was not realized, however, because of line broadening in chloroform due to aggregation (11). For the same reason, LIS reagent-induced shifts were not used in the remainder of this work, although a marked binding preference of the chelate to the C_3 position in bile acids was noted. At the time this manuscript was nearing completion, the ^{13}C spectral assignments of bile acids were published (12) which were identical to our results, if consideration is given to the solvent effects noted; therefore our assignments will not be given here.

Mass spectrometric analysis of methyl cholate and chenodeoxycholate derived from [1-^{13}C, 1,1-$^{2}H_2$] ethanol administration showed an excess of about 220 atoms of heavy isotope per 100 molecules. Results of previous work (3) using [1,1-$^{2}H_2$] ethanol indicated that about 60 of these atoms were the ^{2}H isotope. About 40% of the molecules were entirely unlabeled. Analysis of methyl cholate derived from [2,2,2-$^{2}H_3$] ethanol administration showed a deuterium excess of 280 atoms per 100 molecules. If it is assumed that deuterium is not lost from C_2 of ethanol during cholesterol biosynthesis this corresponds to 17 atoms percent excess in each position expected to be labeled. About 45% of the molecules were unlabeled.

Heavy isotope incorporation at specific carbon sites in rat bile acids, as determined by ^{2}H,^{1}H-decoupled ^{13}C nmr is shown in Table I. Portions of comparative spectra which allowed these measurements are shown in Fig. 1.

DISCUSSION

Evidence is growing that at least for tetrahedral carbon, ^{13}C-^{2}H isotope shifts are predictable, i.e., upfield, and linearly related to the number of directly attached, geminal, and vicinal deuterons (13). The ^{2}H,^{1}H-decoupled

TABLE I

Heavy Isotope Incorporation in Bile Acids[a]

Carbon	^{13}C enrichment[b]		^{2}H incorporation (%)[d], & ^{2}H isotope shift (ppm)[e]		
	1a	2a	1a	2a	1b
1					δ
2	c	13	δ	9, .40	δ
3					0
4	19	18	δ	0	δ
5					δ
6	c	20	δ	δ	δ
7					8, .47
8[k]	19	18	0	0	0
9					0
10[g]	18	c			
11[j]	14	17	8, .36	9, .35	0
12[j]	17	18	δ	10, .36	δ
13[g]					
14[k]	16	19	0	0	0
15					9, .34
16	16	21	12, .36	13, .39	δ
17					6, .47
18					(12, .22)[h]
19					(16, .19)[i]
20	c	c	δ	0	δ
21					6, .86
22					δ
23	24	26	0	3, .27	0
24[g]					

[a] 1a and 2a are methyl cholate and methyl chenodeoxycholate, respectively, from bile collected between 13-18 hr after the beginning of [1-^{13}C, 1,1-$^{2}H_2$] ethanol administration. 1b is methyl cholate from bile collected between 24-48 hr after the beginning of [2,2,2-$^{2}H_3$] ethanol administration. The quantities available for nmr analyses were: 1a and 2a, approximately 3 mg each; 1b, 45 mg.

[b] in multiples of natural abundance, assuming no labeling of carbon atoms derived from C_2 of acetate. To avoid large errors resulting from unequal nuclear Overhauser enhancement (N.O.E.) and spin-lattice relaxation times (T_1), the weak natural abundance ^{13}C resonances of 1a and 2a were integrated, and compared with the integrated intensity of the same resonances of standards observed under identical experimental conditions, resulting in normalization

factors S_{1a} and S_{2a}. The ratios of ^{13}C-enriched intensities in 1a and 2a to their same intensities in the standard spectra were then multiplied by these normalization factors to arrive at the enrichment multiples. Thus, although the absolute values of enrichment are subject to the high experimental uncertainty (±25%) in the natural abundance intensities of 1a and 2a, the relative enrichment of ^{13}C sites is valid to ca. ±5%. Small errors resulting from new N.O.E.'s and T_1's due to partial ^2H incorporation are ignored.

[c] accurate calculation not possible due to overlapping peaks in ^{13}C enriched or standard spectra.

[d] minimum percentage (see discussion) of ^{13}C atoms carrying ^2H atom(s); calculated as the difference in integrated areas of ^2H-shifted peaks between comparable ^2H, ^1H-decoupled and ^1H-decoupled spectra, divided by the integrated ^{13}C present at that site.

[e] all isotope shifts are to higher field.

[f] unobservable due to peak overlap.

[g] non-protonated carbon.

[h] geminal isotope shift of C_{13}.

[i] geminal isotope shift of C_{10}.

[j] $J_{C_{11}C_{12}}$ of 1a, 36.4 Hz; of 2a, 32.4 Hz.

[k] $J_{C_8C_{14}}$ of 1a, 35.6 Hz; of 2a, 36.4 Hz.

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technique is designed to observe the largest of these, i.e., the carbon to which the deuteron is directly attached, so it should be able to detect partial deuteration in methylene and methyl groups quite easily. In fact, in Table I all the methylene groups demonstrating ^2H-incorporation are believed to contain only a single deuteron for this reason.

The most appealing aspect of the ^2H-incorporation detection method described here is that a change in the setting of the ^2H decoupler (i.e., "switching" the $J_{^{13}C^2H}$-coupling on or off) is all that is required to observe the qualitative effect, without additional chemical or instrumental procedures. However, the quantitative measure of ^2H-incorporation at specific carbon sites by our "difference" method (see footnote d, Table I) results in systematically low values since some isotopically shifted intensity is present in ^2H-coupled spectra. Other factors also lead to systematic errors in this direction. Allerhand et al. (14) have reported that carbons in cholesteryl chloride relax mainly through ^{13}C-^1H dipolar interactions. Since ^{13}C-^2H dipolar relaxation is approximately 16 times less efficient, one would expect the relaxation times and ^1H nuclear Overhauser enhancements of individual carbons in partially deuterated molecules to depend on proximity to nearby protons in a complicated manner. In this study, we have not used instrumental or paramagnetic ion methods to suppress nuclear Overhauser enhancement (15,16) or reduce carbon T_1's (16), but in any case the ^2H,^1H-decoupled ^{13}C nmr technique can produce only the lower estimate of ^2H site incorporation. For example, if the non-

deuterated carbon T_1's of C_{18} and C_{19} in methyl cholate are nearly equal to 1.5 sec as in cholesteryl chloride (14), complete deuteration as might be expected in the case of [2,2,2-$^2H_3$] ethanol administration would render these carbons invisible, under the conditions our data were taken, since the nearest protons are 3 bonds removed. This was in fact the case, indicating that there is apparently no exchange of deuterium at ethanol-C_2 on oxidation to acetate and activation to acetyl CoA. The ^2H incorporation at C_{18} and C_{19} was instead detected in the geminal ^{13}C-^2H isotope shifts experienced by C_{10} and C_{13}.

Estimates of full or partial deuterium content should also be gained from the decrease in intensities of residual undeuterated peaks in ^1H-decoupled ^{13}C spectra, from their values in spectra of unenriched standards run under identical conditions. This method would be expected to give systematically low estimates since it ignores overlapping of ^2H-coupled peaks (see also ref. 2), but particularly fails here because it depends on small differences in large numbers (intensities) with relatively large uncertainties. However, when applied to the spectra of methyl cholate derived from [2,2,2-$^2H_3$] ethanol administration, the largest decreases in intensity occurred in the C_7, C_{15}, C_{17}, C_{18} and C_{21} resonances, and suggested that ~15% of each of these carbon atoms was deuterated.

Double (^{13}C,^2H) isotopic incorporation in bile acids from [1-^{13}C, 1,1-$^2H_2$] ethanol results in marked ^{13}C incorporation at 11 carbon sites. If it is assumed that carbon atoms derived from C_2 of acetate are unlabeled, the total ^{13}C excess of the molecules can be calculated from the nmr data to be about 210 atoms percent. Mass spectrometric analysis indicated 160 atoms percent excess. This supports the contention that only carbon atoms derived from C_1 of ethanol are labeled. Deuterium is transferred from ethanol to the 4-pro-R site of NADH and would be detected by our technique only if it were incorporated at one of these carbon atoms after transfer to the NADPH used as cofactor in the biosynthesis. The primary reduction of hydroxymethyl glutaryl-CoA to mevalonate resulted in predictable ^2H incorporation at some of these sites. However, ^2H-incorporation at C_{14} is conspicuously absent, and suggests that the 4-pro-S site of NADPH used in the C_{14} demethylation of lanosterol (17) was unlabeled. The low deuterium content at C_{23} is explained by partial loss during alkaline hydrolysis of the conjugated bile acids.

Since the cyclization of squalene to lanosterol is believed to be symmetric, it is difficult to account for the relative differences in ^{13}C enrichment between C_2 and C_{23} (Table I, also see Fig. 1). A reduction in integrated intensity of the undeuterated C_2 peak not accounted for by a corresponding increase in its ^2H-isotopically shifted resonance because of nuclear Overhauser or T_1 effects is not expected as it is predominantly singly deuterated as evi-

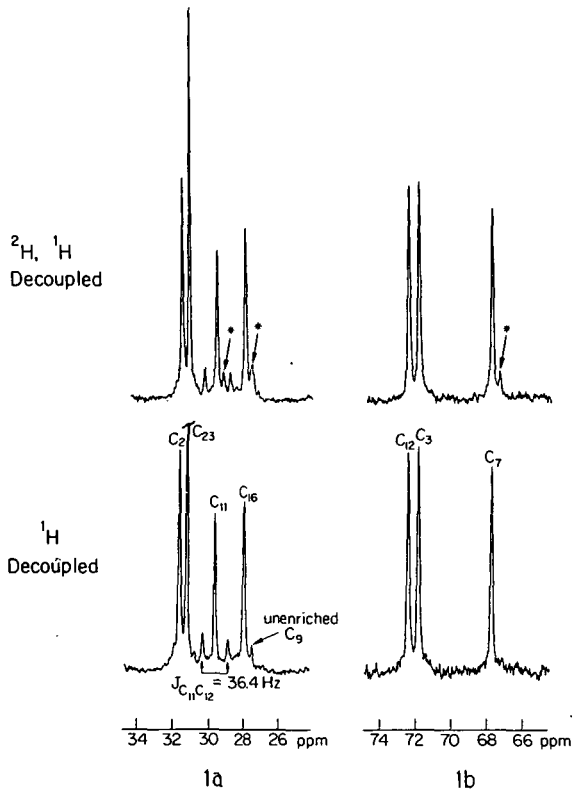

Figure 1. Portions of ^{13}C spectra of (1a) methyl cholate derived from [1-^{13}C, 1,1-$^2H_2$] ethanol administration, and (1b) methyl cholate derived from [2,2,2-$^2H_3$] ethanol administration. Asterisks identify resonances of deuterated carbons, which are separated from their protonated parent peaks at lower field by ^{13}C-^2H isotope shifts. The solvent was pyridine at 50°C. The chemical shift scale is in ppm downfield from internal TMS.

denced by its isotope shift. For the same reason, a large increase in total integrated intensity at C_{23}, because of partial deuterium loss on alkaline hydrolysis, would not be expected. We shortly plan to complement the results given here by investigation of the other 13 carbon sites using [2-^{13}C, 1,1-^2H] ethanol, which may provide some insight into this question.

ACKNOWLEDGEMENT: Financial support from the National Aeronautics and Space Administration Grant NGL 05-003-003 and the Swedish Medical Research Council Grant 13X-2189 is gratefully acknowledged; the authors thank Dr. Peter Christiansen and Ms. Sydell Lewis for the synthesis of labeled ethanol, Miss Birgitta Lindskog for laboratory work and Mr. R. W. Olsen for instrumental design and modification assistance in the performance of this research. One of us (A.L.B.) is grateful to the John Simon Guggenheim Memorial Foundation for a fellowship, 1970-1972.

REFERENCES

1. For reviews of the application of mass spectrometry to the analysis of

stable isotopic incorporation, see Ratner, S. (1972) in Biochemical Applications of Mass Spectrometry (Waller, G. R., Ed.), pp. 1-10, Wiley-Interscience, New York; Caprioli, R. M. (1972) ibid., 735-776; also Grostic, M. F., and Rinehart, K. L., Jr. (1971) in Mass Spectrometry: Techniques and Applications (Milne, G. W. A., Ed.), pp. 217-287, Wiley-Interscience, New York.

2. Stothers, J. B., and Tan, C. T. (1972) J. Amer. Chem. Soc. 94, 8581-8582.
3. Cronholm, T., Burlingame, A. L., and Sjövall, J. (1973), J. Biol. Chem., submitted for publication; also Cronholm, T., Burlingame, A. L., and Sjövall, J. (1973) in Abstracts, Ninth International Congress of Biochemistry, Stockholm, 1-7 July, p. 412.
4. Cronholm, T., Makino, I., and Sjövall, J. (1972) European J. Biochem. 24, 507-519.
5. Hedfjäll, B., Jansson, P.-A., Mårde, Y., Ryhage, R., and Wikström, S. (1969) J. Sci. Instr. 2, 1031-1035.
6. Reimendal, R., and Sjövall, J. (1971) in Hormonal Steroids (James, V.H.T. and Martini, L., Eds.), pp. 228-237, Excerpta Medica, ICS 219, Amsterdam.
7. McPherron, R., Olsen, R. W., and Burlingame, A. L. (1973) in Advances in Mass Spectrometry (West, A. R., Ed.), Institute of Petroleum, London, Vol. 6, in press.
8. Wilson, D. M., Olsen, R. W., and Burlingame, A. L., Rev. Sci. Instr., submitted for publication.
9. Reich, H. J., Jautelat, M., Messe, M. T., Weigert, F. J., and Roberts, J. D. (1969) J. Amer. Chem. Soc. 91, 7445-7454.
10. Mantsch, H. H., and Smith, I. C. P. (1973) Canad. J. Chem. 51, 1384-1391.
11. Bennet, W. S., Eglinton, G., and Kovac, S. (1967) Nature 214, 776-780.
12. Leibfritz, D., and Roberts, J. D. (1973) J. Amer. Chem. Soc. 95, 4996-5003.
13. Colli, H. N., Gold, V., and Pearson, J. E. (1973) J. C. S. Chem. Comm., 408-409, and references therein; Breitmaier, E., Jung, G., Voelter, W., and Pohl, L. (1973) Tetrahedron 29, 2485-2489.
14. Allerhand, A., Doddrell, D., and Komoroski, R. (1971) J. Chem. Phys. 55, 189-198.
15. Freeman, R., Hill, H. D. W., and Kaptein, R. (1972) J. Magnetic Resonance 7, 327-329.
16. Freeman, R., Pachler, K. G. R., and La Mar, G. N. (1971) J. Chem. Phys. 55, 4586-4593; Gansow, O., and Burke, A. (1972) Chem. & Eng. News, April 10, 15.
17. Wilton, D. C., Watkinson, I. A., and Akhtar, M. (1970) Biochem. J. 119, 673-675.